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Genetic Diversity of Human Immunodeficiency Virus Type 2: Evidence for Distinct Sequence Subtypes with Differences in Virus Biology

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The virulence properties of human immunodeficiency virus type 2 (HIV-2) are known to vary significantly and to range from relative attenuation in certain individuals to high-level pathogenicity in others. These differences in clinical manifestations may, at least in part, be determined by genetic differences among infecting virus strains. Evaluation of the full spectrum of HIV-2 genetic diversity is thus a necessary first step towards understanding its molecular epidemiology, natural history of infection, and biological diversity. In this study, we have used nested PCR techniques to amplify viral sequences from the DNA of uncultured peripheral blood mononuclear cells from 12 patients with HIV-2 seroreactivity. Sequence analysis of four nonoverlapping genomic regions allowed a comprehensive analysis of HIV-2 phylogeny. The results revealed (i) the existence of five distinct and roughly equidistant evolutionary lineages of HIV-2 which, by analogy with HIV-1, have been termed sequence subtypes A to E; (ii) evidence for a mosaic HIV-2 genome, indicating that coinfection with genetically divergent strains and recombination can occur in HIV-2-infected individuals; and (iii) evidence supporting the conclusion that some of the HIV-2 subtypes may have arisen from independent introductions of genetically diverse sooty mangabey viruses into the human population. Importantly, only a subset of HIV-2 strains replicated in culture: all subtype A viruses grew to high titers, but attempts to isolate representatives of subtypes C, D, and E, as well as the majority of subtype B viruses, remained unsuccessful. Infection with all five viral subtypes was detectable by commercially available serological (Western immunoblot) assays, despite intersubtype sequence differences of up to 25% in the *gag*, *pol*, and *env* regions. These results indicate that the genetic and biological diversity of HIV-2 is far greater than previously appreciated and suggest that there may be subtype-specific differences in virus biology. Systematic natural history studies are needed to determine whether this heterogeneity has clinical relevance and whether the various HIV-2 subtypes differ in their *in vivo* pathogenicity.

Since the first discovery of human immunodeficiency virus type 2 (HIV-2) in West Africa in the mid-1980s (6, 13), much has been learned about the epidemiology, geographic distribution, and disease association of this second human immunodeficiency virus (for a review, see reference 61). HIV-2 seroprevalence and seroincidence rates have been determined (18, 73, 74, 77, 80, 94), and the spectrum of immunological and clinical consequences of HIV-2 infection has been characterized (19, 20, 62, 67, 68, 92, 93). In addition, numerous virus isolates have been obtained (10, 14, 25, 50, 52, 53, 81), and their molecular and biological properties have been determined (7, 22, 28, 35, 38, 46, 54, 84, 86, 97). Results from these studies indicate that the clinical manifestations of HIV-2 infection are generally similar to those of HIV-1, in that both viruses can cause immunodeficiency and AIDS in infected individuals (61). The two virus types also share structural, genetic, and biological properties and are believed to cause CD4 cell depletion by similar mechanisms (36, 61). Despite these similarities, however, there is increasing evidence that HIV-1 and HIV-2 differ in their natural history of infection

and *in vivo* pathogenicity (18, 62, 72, 76). HIV-2-infected individuals exhibit longer clinical latency periods (4, 24), progress more slowly after onset of symptoms (62, 76), and, in the clinically asymptomatic stage, appear to have a lower virus burden than individuals infected with HIV-1 (83). In addition, both vertical and horizontal transmission rates are significantly lower for HIV-2 (5, 23, 45). Finally, HIV-2 is relatively confined to West Africa, and its appearance in other geographic areas has generally reflected epidemiological links to individuals of West African origin (18, 75).

Clinical and biological differences between the two human AIDS viruses must be viewed in the context of their origins and evolutionary histories. Nonpathogenic lentiviruses (simian immunodeficiency viruses [SIVs]) related to HIV-1 and HIV-2 have been identified in different Old World primate species, and phylogenetic analyses of their sequences indicate that they fall into five major lentivirus lineages (SIV_{CPZ} from chimpanzees, SIV_{SM} from sooty mangabeys, SIV_{SYK} from Sykes' monkeys, SIV_{AGM} from African green monkeys, and SIV_{MND} from mandrills; see reference 82 for a review). Representatives of the two human viruses do not form independent lineages but instead cluster with either the chimpanzee viruses (HIV-1) or the sooty mangabey viruses (HIV-2) (42, 44). These phylogenetic relationships, along with epidemiological and virological

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data arguing for a simian origin of HIV (32, 42, 44, 64, 70), have led to the conclusion that HIV-1 and HIV-2 are the result of simian-to-human cross-species transmissions and represent zoonotic infections of humans (66, 82). In light of these findings, differences in HIV-1 and HIV-2 biology and natural history should not be surprising. Rather, such differences should be expected, given the fact that the predecessors of HIV-1 and HIV-2 evolved in diverse primate hosts, probably for long periods of time, before they entered the human population.

Evaluation of the full spectrum of HIV-1 and HIV-2 genetic diversity represents an important first step in the elucidation of their biological heterogeneity and varying virulence properties. Over the past several years, much effort has been focused on characterizing the global diversity of HIV-1 (21, 31, 60, 65). Extensive molecular epidemiological studies have shown that HIV-1 strains fall into two major genetic clusters, which appear to represent independent introductions of primate lentiviruses into the human population (82). One cluster (group M) comprises the vast majority of HIV-1 isolates (including all those characterized during the first decade of HIV-1 research) and can be further subdivided into eight distinct lineages on the basis of equidistance in the *env* and *gag* regions (60, 65). These lineages have been termed sequence subtypes A to H (60, 65). The second cluster (group O) was only recently discovered and is thus far represented by only a small number of viruses from Cameroon (34, 88). Viruses from all these different lineages have been identified in AIDS patients, and they are generally believed to exhibit similar biological and pathogenic properties. This assumption, however, may be premature, since systematic studies comparing their natural history and in vivo pathogenicity have not yet been performed.

In comparison to HIV-1, HIV-2 genetic variation has appeared to be more limited. Only two sequence subtypes are currently identified in the database (65), which, by analogy to HIV-1, have been termed A and B. The great majority of characterized HIV-2 strains, including all initially reported (prototypic) isolates (ROD, ST, BEN, ISY, etc.), cluster in sequence subtype A. Subtype B viruses are less frequent, and only three representatives have thus far been reported (D205, GH-2, and UC-1) (7, 22, 46). Importantly, subtype classification of HIV-2 is based almost exclusively on sequences derived from tissue culture-amplified isolates. Arguing that such isolates might represent only a subset of a larger, genetically more complex group of viruses, we reported previously the characterization of HIV-2 sequences obtained directly from uncultured patient material (32). Using nested PCR techniques, we amplified the *env* and *pol* regions of three unique viruses. Phylogenetic analyses suggested that one of these (7312A) was a recombinant of subtype A and B viruses. The two others, both derived from individuals from whom virus isolation was repeatedly unsuccessful, were quite distinct from all other known HIV-2 strains. One (2238) was somewhat more closely related to subtype B than to subtype A viruses, although highly divergent from both; the other (FO784) was more closely related to SIVs infecting sooty mangabeys (SIV_{SM}) and macaques (SIV_{MAC}) than to viruses of human derivation. Although these results provided evidence for the existence of additional evolutionary lineages, definitive subtype classification of FO784 and 2238 was not possible, primarily because of the limited number of non-subtype A HIV-2 sequences in the database (only 10 full-length HIV-2 sequences have thus far been reported, 8 of which represent viruses that belong to sequence subtype A [65]).

Following up on these earlier studies, we report here the PCR amplification and sequence analysis of the *gag*, *pol*,

reverse transcriptase (RT), and *env* fragments from the peripheral blood of nine additional HIV-2-infected individuals. Analyses of these new viruses, along with more extensive characterization of the previously reported 2238, FO784, and 7312A strains (32), have now allowed a comprehensive study of HIV-2 phylogeny and have revealed the existence of five major lineages, which we term HIV-2 sequence subtypes A to E. Importantly, viruses representing the new sequence subtypes (C to E) were all derived from individuals from whom virus isolation was repeatedly unsuccessful. These results thus indicate that analyses of viruses that grow readily in tissue culture may substantially underrepresent viral strains present in certain human populations.

MATERIALS AND METHODS

Subjects. Table 1 summarizes available demographic and clinical information for all study participants. Patient histories are described in greater detail below except for subjects 2238, FO784, 7312A, and PA, which have been reported previously (32, 43).

Subjects ON, JA, FT, and FA were identified to be HIV-2 infected at the University of Ghana Medical Center (Korle Bu Hospital) in Accra. Three of them (ON, JA, and FA) were in-patients and suffered from end-stage AIDS. Patient ON was a 48-year-old fisherman who was hospitalized because of weakness, fever, weight loss, chronic diarrhea, and oral thrush. As risk factors for infection, he reported frequent encounters with prostitutes in Ghana as well as in Côte d'Ivoire. Subject FT was his wife, who was also infected with HIV-2 but clinically asymptomatic. Patient JA was a 27-year-old female who suffered from chronic diarrhea, severe weight loss, anemia, and alopecia. She was married to a Nigerian man and had lived in Lagos, Nigeria, for 5 years prior to returning to Ghana because of onset of illness. Patient FA was a 36-year-old female who was admitted to the hospital because of pulmonary tuberculosis. She had lived in both Ghana and Côte d'Ivoire for several years and suffered from fever, severe weight loss, diarrhea, and AIDS dementia complex.

Subjects 7810A, 7924A, 60667K, and 60415K were identified as HIV-2 seropositive in the United States. All of them were heterosexual West African nationals living in the Washington, D.C., area, where they sought medical care for sexually transmitted diseases (STDs). Moreover, all of them originated from urban areas of West Africa and had traveled extensively before coming to the United States. Subject 7810A was a 31-year-old student from Côte d'Ivoire. He was asymptomatic at the time of presentation, with normal CD4 counts, and a heterosexual partner of 3 years was found to be HIV seronegative. Subject 7924A was a 48-year-old teacher from Guinea Bissau who was hospitalized because of severe weight loss and chronic diarrhea. Clinical evaluation revealed reduced CD4 counts (50 cells per mm³) and infection with *Shigella flexneri* and *Giardia lamblia*. Subject 60667K was a 37-year-old female from Sierra Leone who presented with low CD4 counts (361 cells/mm³) but was otherwise asymptomatic. She had two teenage children, who were both HIV seronegative. Subject 60415K was a 37-year-old asymptomatic man from urban Senegal who was married to an American intravenous drug abuser.

Serology. Patients were identified as HIV-2 seroreactive (or HIV dually reactive) by collaborating investigators. All serological results were confirmed with commercially available Western immunoblot kits (NEW LAV-BLOT I/II and PEPTI-LAV 1-2 from Pasteur Diagnostics, Marnes-la-Coquette, France; HIV-1 and HIV-2 Western Blots from Cambridge Biotech, Worcester, Mass.). Assays were performed and re-

TABLE 1. Demographic and clinical characteristics of the study participants^a

Subject	Age (yr)	Sex	Origin	Disease status	CD4 count (cells/mm ³)	Blood sample date(s) (mo/yr)	Serology
2238	47	Male	Liberia (rural)	Asymptomatic	N/A	1/89*	HIV-2
FO784	46	Male	Liberia (rural)	Asymptomatic	N/A	5/89 11/89 3/90*	HIV-2
ON	48	Male	Ghana (urban)	AIDS	N/A	5/90*	Dual HIV
FT	N/A	Female	Ghana (urban)	Asymptomatic	N/A	5/90*	HIV-2
JA	27	Female	Ghana (urban)	AIDS	N/A	5/90*	Dual HIV
FA	36	Female	Ghana (urban)	AIDS	N/A	5/90*	HIV-2
7312A	32	Male	Côte d'Ivoire (urban)	Lymphadenopathy	587	9/90 6/91*	Dual HIV
7810A	31	Male	Côte d'Ivoire (urban)	Asymptomatic	769	5/93*	HIV-2
7924A	48	Male	Guinea-Bissau (urban)	AIDS	50	7/93*	Dual HIV
60415K	34	Male	Senegal (urban)	Asymptomatic	698	7/93*	HIV-2
60667K	37	Female	Sierra Leone (urban)	Asymptomatic	361	4/93*	Dual HIV
PA	35	Male	Sierra Leone (rural)	Asymptomatic	>500	3/91* 10/91	HIV-2

^a Informed consent was obtained from all study participants prior to blood collection. Patient histories are described in Materials and Methods except for subjects 2238, FO784, 7312A, and PA, which have been reported previously (32, 43). Asterisks denote serum or plasma samples used for serological confirmation (compare Fig. 4). N/A, information not available.

sults were scored according to the manufacturer's recommendations.

Tissue culture. Peripheral blood from subjects ON, FT, JA, and FA was collected in Ghana and transported to the United States within 48 h of phlebotomy. Blood samples from 7810A, 7924A, 60415K, 60667K, and PA were obtained in the United States. Blood was processed and cultured in four different laboratories (Table 2): 7810A, 7924A, 60415K, and 60667K at the University of Alabama at Birmingham (UAB); ON, FT, JA, and FA at the National Cancer Institute (NCI, Bethesda, Md.); and PA at the University of California, Los Angeles (UCLA, Los Angeles, Calif.) and the Aaron Diamond AIDS Research Center (ADARC, New York, N.Y.). Culture attempts from subjects 2238, FO784, and 7312A (performed at UAB) have been reported previously (32); the results are included in Table 2 for comparison.

Plasma was separated by low-speed centrifugation, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation as described before (12, 15, 78). A portion of the cells was used for DNA extraction (see below), and the remainder was cultured by different isolation ap-

proaches (see Table 2). All PBMC samples were cocultured with phytohemagglutinin-stimulated normal donor lymphocytes according to standard protocols (12, 15, 78). For four patients, this was done on more than one occasion (Table 2). In some instances, virus isolation attempts also included plasma cultures (78) or cocultivation of patient PBMCs with immortalized T-cell lines (CEM, H9, and Molt4 clone 8), normal donor macrophages (prepared by plastic adherence and grown in the presence of recombinant granulocyte-macrophage or macrophage colony-stimulating factor [57]), and rhesus macaque PBMCs (10). On two occasions, lymphocyte preparations from patient PA were fractionated prior to culture to remove CD8⁺ cells. This was done by causing patient PBMCs to adhere to plastic dishes coated with CD8-specific antibodies as described before (96). All cultures were maintained for several weeks, inspected for the appearance of virus-induced syncytia, and monitored for supernatant RT activity or the presence of p24/p27 antigen (12, 15, 78). Cultures were considered positive when RT activity or p24/p27 antigen was detectable on two consecutive occasions.

PCR. High-molecular-weight DNA was extracted from un-

TABLE 2. Attempts to isolate virus from the peripheral blood of 12 HIV-2 seropositive individuals^a

Subject	Laboratory	No. of independent isolation attempts	Cocultivation of patient PBMCs with:				Plasma culture	CD8 depletion of patient PBMCs
			Normal donor PBMCs	Normal donor macrophages	Immortalized T-cell lines	Rhesus macaque PBMCs		
2238*	UAB	2	—	ND	—	ND	—	ND
FO784*	UAB	2	—	—	—	ND	—	ND
ON	NCI	1	—	ND	ND	ND	ND	ND
FT	NCI	1	—	ND	ND	ND	ND	ND
JA	NCI	1	—	ND	ND	ND	ND	ND
FA	NCI	1	+	ND	+	ND	ND	ND
7312A*	UAB	3	+	ND	+	ND	—	ND
7810A	UAB	1	—	ND	ND	ND	ND	ND
7924A	UAB	1	+	ND	ND	ND	ND	ND
60415K	UAB	1	+	—	ND	ND	ND	ND
60667K	UAB	1	+	ND	ND	ND	ND	ND
PA	UCLA and ADARC	7	—	—	—	—	—	—

^a Virus isolation was performed at UAB, NCI, UCLA, and ADARC as described in Materials and Methods. Results for subjects 2238, FO784, and 7312A (indicated by asterisks) have been reported previously (32). +, virus-positive culture; —, virus-negative culture; ND, cultures not performed because of lack of patient material.

TABLE 3. Nested PCR primer pairs used to amplify HIV-2 subgenomic fragments from primary PBMC DNA^a

Primer pair	Nucleotide sequence	Location	Reaction conditions ^b	Fragment size (bp)	Genomic region
Outer pair					
LTR A	5'-CTGAGACTGCAGGGACTTTCCAGAAGGG-3'	9379-9406	1 min, 94°C; 1 min, 45°C;	140	LTR (U3/R)
LTR B*	5'-AAGCAGAAAGGGTCCTAACAGACCAGGGT-3'	9739-9767	1 min, 72°C; 30 cycles		
Inner pair					
LTR C	5'-AGGCTGGCAGATTGAGCCCTGGGAGGTTC-3'	9513-9541			
LTR D*	5'-CCAGGCGGCGACTAGGAGAGATGGGAGCAC-3'	9682-9711			
Outer pair					
env A*	5'-GCTAGGGTTCTTTGGGTTTCTCGCGACAGCAGG-3'	7691-7723	1.5 min, 94°C; 1.5 min, 45°C;	453	env (gp41)
env B*	5'-CAAGAGGCGTATCAGCTGGCGGATCAGGAA-3'	8415-8444	1.5 min, 55°C; 35 cycles		
Inner pair					
env C	5'-GGGATAC <u>TCGAC</u> CAACAGCAACAGCTGTTG-3'	7782-7811			
env D	5'-GGGAGGGGAAGAG <u>GAATTC</u> TGGCCTATA-3'	8265-8291			
Outer pair					
pol A*	5'-AATATACTAGTAGATTACAAATATGT-3'	3857-3882	1 min, 94°C; 1.5 min, 40°C;	708	pol (integrase)
pol B*	5'-CTGCCTTCTCTGAAATAGACCCGAAAA-3'	4723-4749	2 min, 70°C; 30 cycles		
Inner pair					
pol A*	5'-AATATACTAGTAGATTACAAATATGT-3'	3857-3882			
pol D*	5'-CTTCTTTTAAAAATTCATGCAATGAACTGCC-3'	4591-4620			
Outer pair					
gag A	5'-AGGTTACGGCCCCGGCGAAAGAAA-3'	603-627	1 min, 94°C; 1 min, 40°C;	781	gag (p16/p28)
gag B	5'-CCTACTCCCTGACAGGCGTCAGCATTTCTTC-3'	1581-1612	2 min, 72°C; 30 cycles		
Inner pair					
gag C	5'-AGTACATGTTAAACATGTAGTATGGGC-3'	628-655			
gag F	5'-CCTTAAGCTTTTGTAGAATCTATCTACATA-3'	1437-1466			
Outer pair					
RTC*	5'-ATGACAGGGGATCCCCAATCAATATTTTGG-3'	2309-2339	1.5 min, 94°C; 1 min, 40°C;	995	pol (RT)
RT2	5'-GAAGTCCAGTCTGGGATCCATGTCATTGCCA-3'	3593-3526	2 min, 72°C; 30 cycles		
Inner pair					
RT3	5'-GAGGCATTAAAAGAGATCTGTGAAAAATGG-3'	2474-2504			
RT4	5'-TCCCCAAATGACTAGTGCTTCTTTTCCTAT-3'	3500-3529			

^a Primer pairs were designed according to HIV/SIV consensus sequences (65); sequences are numbered according to the published sequence of HIV-2/ROD (35); asterisks denote previously published primers (restriction enzymes sites are underlined) (3, 32).

^b Conditions used for both sets of primers.

cultured patient PBMCs under PCR cleanroom conditions and subjected to nested PCR amplifications with primer pairs designed according to HIV-2/SIV_{SM}/SIV_{MAC} consensus sequences (65). All primer sequences and amplification conditions are described in Table 3, except for a previously reported set of HIV-1 primers used to exclude HIV-1 infection in individuals with dual HIV seroreactivity (HIV-1 LTR I-IV [3]). PCRs were carried out in a total volume of 100 µl, containing 1 µg of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 pmol of each primer, 200 µM each of the four deoxynucleoside triphosphates (dNTPs), 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, and 2.5 U of *Taq* polymerase. Samples were overlaid with 100 µl of mineral oil to prevent evaporation and then subjected to 30 to 35 amplification cycles, as indicated in Table 3. Second-round PCR products were visualized by agarose gel electrophoresis, isolated from a preparative gel, purified with GeneClean (Bio 101 Inc., La Jolla, Calif.), and subcloned into M13mp18 and 19 by using restriction enzyme sites (*env* fragments) or pCR vectors (Invitrogen, San Diego, Calif.) by T/A overhang (*pol* fragments were subcloned into pCR1000; *gag* and RT fragments were subcloned into pCRII).

DNA sequence analysis. Recombinant clones containing HIV-2 fragments were sequenced manually by the dideoxynucleotide chain termination method (Sequenase Kit; US Biochemicals, Cleveland, Ohio) or on an automated DNA sequenator (model 373A; Applied Biosystems, Inc.) using cycle

sequencing and dye terminator methods. Generally, only one clone per amplification product was sequenced except for *env* and *pol* fragments from subjects ON, JA, and FA and *gag* and RT fragments from subject FO784, for which multiple sequences were obtained (data not shown). Importantly, 7312A *gag*, *pol*, and *env* sequences were not derived from PCR products but from a replication-competent provirus cloned from a short-term-infected PBMC culture (37). This was done to examine previously noted discordant phylogenetic positions of 7312A *env* and *pol* sequences (32) in the context of a single viral genome. Sequence analysis was performed with PC/Gene (IntelliGenetics Inc., Mountain View, Calif.), EuGene (Baylor College of Medicine, Houston, Tex.), MASE (26), and DOTS (55).

Phylogenetic analysis. The phylogenetic relationships of the newly identified viruses to previously published HIV-2/SIV_{SM}/SIV_{MAC} strains (65) were estimated from comparisons of their nucleotide sequences. Sequences were aligned by using CLUSTAL (39, 40) with minor manual adjustments, bearing in mind their predicted protein sequences. Pairwise evolutionary distances were estimated by using Kimura's two-parameter method (equation 4.14 in reference 49) to correct for superimposed substitutions. Sites at which there was a gap in any of the aligned sequences were excluded from all comparisons. Phylogenetic relationships were computed from these distances by the neighbor-joining method (79). The repeatability

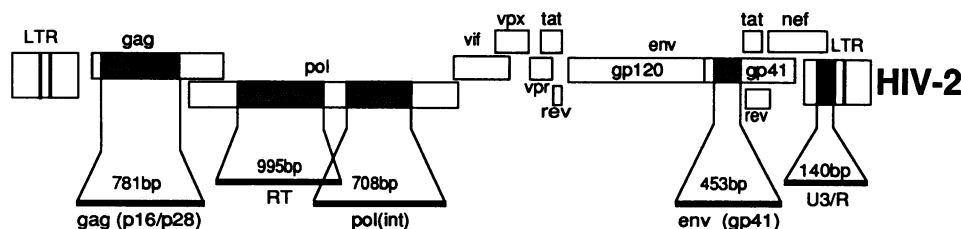


FIG. 1. Location of HIV-2 sequences amplified from uncultured patient PBMCs. Sequences derived from the *gag* (781 bp), *pol* (RT region, 995 bp; integrase [int] region, 708 bp), and *env* (453 bp) regions were used for phylogenetic analyses; the LTR (140 bp) region was amplified for diagnostic purposes only.

of the branching orders obtained was estimated by the bootstrap approach (27). These methods were implemented with CLUSTAL V (39). Phylogenetic relationships were also analyzed from predicted protein sequences (using Kimura's empirical method to estimate distances; equation 4.8 in reference 49) and by using alternative approaches to phylogenetic reconstruction, such as the dynamically weighted parsimony method (95).

Nucleotide sequence accession numbers. All sequences were submitted to GenBank and the Los Alamos Database. Clone designations and GenBank accession numbers are listed below (clone designations contain patient identifier, genomic region, and clone number): 60415Kgag.8, L33076; 2238gag.1, L33077; 60667Kgag.1, L33078; 7312Agag.JK, L33079; 7810Agag.2, L33080; 7924Agag.1, L33081; FAgag.2, L33082; FO784gag.10/3, L33083; FTgag.1, L33084; JAgag.1, L33085; ONgag.18, L33086; PAgag.1, L33087; 7312Apol.JK, L33088; FApol.1, L33089; JApol.9, L33090; ONpol.6, L33091; 7312ART.JK, L33092; PART.6, L33093; 7312Aenv.JK, L33094; FAenv.15, L33095; JAenv.12, L33096; and ONenv.12, L33097.

RESULTS

Demographic and clinical characteristics of the study participants. The purpose of this study was to determine the extent of HIV-2 genetic and biological heterogeneity as it exists in infected individuals in vivo. To this end, we collected blood samples from 12 HIV-2 seropositive individuals and characterized their viruses by PCR amplification of viral sequences directly from uncultured PBMC DNA. Although the samples were not obtained as part of a systematic molecular epidemiological survey, they were nevertheless analyzed in a systematic fashion; that is, molecular characterization was performed on all specimens with serological indication of HIV-2 infection regardless of whether simultaneous attempts to isolate virus were successful. Moreover, viruses were analyzed from a geographically diverse group of individuals (Table 1). The 12 subjects originated from six different West African countries and represented urban as well as rural populations. Finally, virus was characterized from individuals at different stages of disease. Seven subjects were healthy at the time of blood collection, while five others had symptoms ranging from generalized persistent lymphadenopathy to end-stage AIDS. The viruses reported in this study are thus representative of a broad spectrum of HIV-2 infections and are not biased with respect to in vitro replication potential, geographic origin, or disease association.

Virus isolation studies. Virus isolation was performed in four different laboratories (UAB, NCI, UCLA, and ADARC) by techniques routinely employed to culture HIV-1 from individuals at all stages of infection (12, 15, 78). Table 2 summarizes the different strategies used as well as the number

of independent isolation attempts (culture attempts for subjects 2238, FO784, and 7312A have been reported previously, but the results are included in Table 2 for comparison [32]). All patient PBMCs were cocultured with phytohemagglutinin-stimulated normal donor lymphocytes according to standard protocols (12, 15, 78). In some cases, isolation attempts also included cocultivation with normal donor macrophages (57) and immortalized T-cell lines (52), as well as plasma cultures (78) (Table 2). Using these approaches, virus was successfully recovered from primary PBMC cultures of subjects FA, 7924A, 60415K, and 60667K. Moreover, virus from subject FA replicated to high titers and was transmissible to the immortalized T-cell line CEM. By contrast, virus culture from blood samples of subjects ON, FT, JA, 7810A, and PA remained unsuccessful. This was the case despite active cell replication, excellent cell viability, and, in some cases, confirmation of HIV-2 viral sequences in short-term-cultured PBMC DNA by PCR amplification.

One of the subjects from whom virus isolation was repeatedly unsuccessful was patient PA. This immigrant from rural Sierra Leone was identified as HIV-2 seropositive following clinical evaluation for kidney transplantation because of end-stage obstructive uropathy (43). Although the source of transmission remained unknown, patient history revealed that subject PA had likely acquired his HIV-2 infection some 20 years ago, prior to his move to the United States (43). Because the patient underwent weekly dialysis at a Los Angeles hospital, blood samples were frequently available and did not require long transportation times (blood was usually hand carried to the laboratory). Thus, additional isolation strategies were pursued. These included multiple cocultivation attempts with normal donor lymphocytes (using up to 10^7 patient PBMCs per individual culture), normal donor macrophages, or rhesus macaque PBMCs, as well as the analysis of viral growth following stimulation of PBMC cultures with tumor necrosis factor alpha or granulocyte-macrophage colony-stimulating factor. Moreover, patient PBMCs were fractionated on two different occasions to deplete CD8⁺ cells. This method has been shown to increase isolation efficiencies of HIV-1 by removing cells that produce a factor(s) that suppresses viral replication (91). Between 1989 and 1993, blood was obtained from subject PA on seven independent occasions. Despite these intensive efforts, attempts to isolate virus from subject PA remained unsuccessful.

PCR amplification of HIV-2 sequences from primary PBMC DNA. As an alternative to virus culture, nested PCR was used to characterize viral sequences directly in uncultured patient materials. In a first step, PBMC DNAs were amplified with long terminal repeat (LTR) primers known to detect HIV-2 sequences with high sensitivity and specificity (32, 37). Using this "diagnostic" primer set, PCR products were amplified from all PBMC specimens, confirming HIV-2 infection in all

gag (p16/p28)

p16 ↔ p28

CONS	aNeLDrFGLaESLLeKEGCqkIlsVLp1VPTGSENKSL?NtvcVwCiHAEKvK?TEeAKqivq?RHLvaeTgtaeKMP?tsrPTAppSgrggNyPVQq??gGNYvH	
FA	ITN-----QF-----L-----D-----RKLI-----N-----F-----TG-----	
60415K	-----R-----T-----D-----F-----A-----D-----G-----S-----E-----F-----H-----V-----T-----	
7924A	-----R-----I-----D-----F-----V-----D-----K-----N-----V-----S-----I-----T-----	
ON	V-----R-----H-----T-----F-----Y-----L-----D-----KMA-----S-----VDA-----T-----A-----VA-----	
FT	V-----T-----H-----T-----F-----F-----L-----Q-----D-----KA-----S-----VDA-----V-----A-----KS-----VA-----	
JA	V-----T-----H-----T-----F-----F-----VY-----L-----D-----KA-----S-----AVN-----T-----A-----VA-----	
# 7312A	-----N-----T-----M-----F-----L-----K-----D-----KLA-----S-----VD-----T-----TADK-----A-----TS-----VA-----	
7810A	-----T-----G-----H-----TA-----F-----VY-----L-----D-----KA-----S-----AN-----A-----K-----VA-----	
60667K	V-----E-----N-----G-----H-----A-----E-----F-----Y-----L-----G-----D-----KA-----S-----GN-----A-----K-----S-----G-----IA-----S-----	
F0784	-----N-----N-----Y-----L-----D-----G-----K-----A-----S-----V-----D-----S-----D-----R-----VA-----	
2238	-----A-----T-----T-----E-----Y-----T-----L-----Q-----H-----RNEV-----E-----A-----KN-----A-----S-----GR-----E-----VA-----	
PA	-----R-----N-----HR-----T-----E-----M-----F-----A-----VY-----L-----AV-----D-----KH-----QH-----GGK-----T-----L-----PQ-----I-----N-----	
CONS	lPLSPRTLNAAWVKLvEeKKFGAEVVPgFQALSEGCTPyDINQmLNCVGdHQAAMQIireIIEEAAdWD?qHpppgPlpaGQLRePrGSDIAGTTStv?EQIqWMyRqqNP	
FA	V-----I-----D-----L-----A-----I-----D-----D-----D-----A-----	
60415K	I-----V-----I-----E-----V-----I-----D-----D-----F-----P-----	
7924A	V-----I-----V-----VA-----I-----D-----D-----E-----F-----A-----	
ON	V-----I-----O-----I-----D-----D-----E-----P-----	
FT	V-----I-----O-----I-----D-----D-----E-----P-----	
JA	V-----I-----O-----V-----D-----D-----E-----A-----	
# 7312A	V-----E-----O-----V-----D-----D-----E-----G-----S-----	
7810A	-----D-----E-----O-----S-----M-----D-----E-----A-----	
60667K	-----D-----EN-----IE-----E-----O-----S-----M-----D-----C-----A-----	
F0784	-----D-----E-----D-----EH-----V-----D-----D-----E-----A-----	
2238	H-----T-----L-----E-----D-----EH-----V-----D-----D-----E-----A-----	
PA	S-----E-----V-----RGQ-----Q-----D-----S-----PA-----E-----NP-----	
CONS	?PVGnIYrRWIqIGLQKCVrmYNptnILDvkQGPKepFQS	253
FA	V-----V-----I-----V-----S-----	261
60415K	V-----I-----I-----I-----S-----	260
7924A	V-----I-----I-----S-----	260
ON	V-----I-----S-----	255
FT	V-----I-----S-----	255
JA	V-----I-----I-----E-----	255
# 7312A	I-----I-----	255
7810A	I-----I-----	255
60667K	V-----I-----I-----T-----N-----	255
F0784	I-----I-----I-----T-----	260
2238	V-----D-----S-----	260
PA	I-----D-----S-----	260

RT (reverse transcriptase)

CONS	keGQLEeAPPTNPYNTPTFAIKkkDkNKWRmLIDFRELNkVtQDFTevqlgiPhpaglAkkRritvIdvGDAYFSIPLde?FRqYTAFTLPsvNNAEPgKRYiYKVlPQGW	
# 7312A	-----S-----S-----E-----WPE-----I-----V-----PD-----I-----	
F0784	R-----S-----S-----V-----R-----KE-----T-----R-----	
PA	-----R-----S-----A-----KEME-----I-----V-----PE-----I-----Q-----	
CONS	KGSpaIfqytMR?vLePFRkANpDviliCYMDDLLIASDRtdLEHDrVVlQlKElLN??GFstPeEKfQKDPpfgWMGyELWptkWKlQkiqLPqke?WTVNdIQKLVGVL	
# 7312A	-----S-----K-----D-----R-----N-----TI-----V-----S-----K-----S-----DM-----K-----I-----ER-----V-----	
F0784	-----NI-----S-----T-----L-----G-----N-----SMT-----M-----V-----DM-----NL-----D-----RE-----LK-----L-----Y-----K-----E-----R-----D-----	
PA	-----V-----E-----Q-----S-----L-----L-----G-----N-----SMT-----M-----V-----DM-----NL-----D-----RE-----LK-----L-----Y-----K-----E-----R-----	
CONS	NWAAQIypGIKTKhLrCrLirGKMt1TEEVQWTElAEAEleENKIIlsQeqEG?YYqE?kplEAtv?K?gdnQW?YKiHQe?kiLkVgK?AKiKnthtngvrLLAhVvQK	319
# 7312A	-----LF-----R-----I-----K-----MQ-----G-----S-----K-----GV-----Q-----NLA-----T-----GN-----V-----Y-----V-----I-----AhVvQK	331
F0784	-----K-----K-----M-----Y-----C-----S-----V-----D-----S-----D-----Y-----Y-----V-----I-----AhVvQK	331
PA	-----V-----N-----K-----S-----A-----G-----R-----R-----EEN-----L-----N-----S-----GDR-----FPQ-----S-----	331

pol (RNase H/integrase)

RNase H ↔ integrase

CONS	MGIvaggPtEsEsrlVnqIIeEMIKKeaiYVaVVPAAHkgIGmQEvDHLVSQgIRQVLFlekIEPAQEEHeKYHsnvKeLvHKfGlpP?lVArQIVntCdkcqKGEaIHGQ	
FA	V-----N-----I-----D-----G-----L-----R-----A-----I-----Q-----T-----I-----AQ-----	
ON	I-----T-----PI-----K-----G-----L-----G-----I-----Q-----K-----S-----V-----	
JA	I-----T-----PI-----K-----G-----L-----G-----I-----Q-----K-----S-----V-----	
# 7312A	I-----T-----PI-----K-----G-----L-----G-----I-----Q-----K-----S-----V-----	
F0784	I-----T-----PI-----K-----G-----L-----G-----I-----Q-----K-----S-----V-----	
2238	L-----I-----T-----P-----R-----L-----R-----V-----IS-----L-----D-----RF-----A-----T-----I-----N-----K-----HV-----	
CONS	vNaelGtWQMDcTHLEGIiIVAVHVaSGFIEAEVipQEtGRQTALFLKLAsRWPiTHLHTDNGaNFtSqeVKMvAWW?gIEqtFgVPYNPQSQgVVEAMNHLKKnQIdR	
FA	I-----V-----K-----KSE-----R-----S-----D-----V-----S-----	
ON	-----V-----D-----V-----R-----	
JA	-----V-----D-----V-----R-----	
# 7312A	-----V-----D-----V-----R-----	
F0784	-----V-----D-----V-----R-----	
2238	D-----V-----A-----V-----V-----H-----K-----	
CONS	iReQAns?ETiVL	232
FA	-----TI-----	235
ON	D-----V-----L-----V-----	235
JA	D-----V-----I-----V-----	235
# 7312A	D-----V-----I-----V-----	235
F0784	D-----V-----I-----V-----	235
2238	D-----TM-----	235

env (gp41)

extracellular

CONS	DVVKRQQEILRLTVWGtKnLQtRVtAIEKYLkdQAqlNsWGCAFRQVCHTtVPwpNdsLtPdWnNmtWQeWE?kv?fLeaNit?lLeEAQIQEQEkNmYELQKlNswDvfgN	
FA	M-----A-----L-----ETIE-----Q-----QIIRY-----SES-----Q-----	
ON	-----L-----ETIE-----Q-----Q-----D-----Q-----Q-----S-----	
JA	-----L-----ETIE-----Q-----Q-----D-----Q-----Q-----S-----	
# 7312A	M-----A-----L-----ETIE-----Q-----Q-----D-----Q-----Q-----S-----	
F0784	-----M-----A-----L-----ETIE-----Q-----Q-----D-----Q-----Q-----S-----	
2238	M-----K-----E-----ST-----Q-----Q-----D-----Q-----Q-----S-----	
CONS	domain ↔ membrane spanning domain	
FA	WfDltsWIKYIqYgyivvgvi?LRiviYivQmLarLRK	147
ON	-----V-----I-----VA-----I-----V-----S-----R-----	151
JA	-----F-----MAHLRL-----L-----V-----A-----I-----V-----M-----	151
# 7312A	-----F-----MA-----KL-----L-----V-----A-----L-----V-----I-----K-----	151
F0784	-----A-----V-----I-----VA-----VI-----V-----IG-----R-----	151
2238	-----L-----V-----YL-----F-----A-----V-----A-----V-----M-----	151

study subjects. By contrast, diagnostic HIV-1 LTR primers (3) failed to amplify viral sequences, excluding simultaneous coinfection with HIV-1 in individuals with dual seroreactivity (Table 1).

Nested PCR techniques were then used to derive larger genomic regions for sequence determinations and phylogenetic studies. Four independent and nonoverlapping regions were amplified (Fig. 1), two of which (a 451-bp *env* gp41 and a 708-bp *pol* integrase fragment) were chosen to allow comparison with previously reported FO784 and 2238 sequences (32). The other two, a 781-bp *gag* (p16/p27) and a 995-bp *pol*(RT) fragment, were selected to obtain additional sequences for a more comprehensive phylogenetic analysis. By using the primers and conditions summarized in Table 3, all 12 PBMC specimens were amplified in at least one of the four targeted regions (because of lack of patient material, not all PBMC DNAs could be amplified with all primer pairs). There were only three instances in which primer pairs failed to amplify the corresponding HIV-2 fragment despite repeated attempts and various amplification conditions. These involved *env* and *pol* (integrase) regions from subjects PA and FT and the *pol* (RT) region from subject 2238.

Figure 2 depicts alignments of deduced amino acid sequences from available PCR fragments. Only sequences derived from the 12 study subjects are shown in comparison to the HIV-2/SIV_{SM}/SIV_{MAC} consensus sequence obtained from the database (65). Previously reported *env* and *pol* sequences from 2238 and FO784 as well as RT sequences from FO784 are indicated by asterisks (32). Most sequences were derived from single PCR clones except for *env*, *pol*, and RT amplification products for ON, JA, FA, and FO784, for which multiple clones were sequenced (not shown). In the latter cases, the predominant clone which also contained an uninterrupted open reading frame was chosen for comparison (Fig. 2 and 3). Finally, all 7312A sequences were derived from a full-length proviral genome cloned as a recombinant lambda phage from short-term-cultured PBMC DNA (37). This was done to examine the previously noted discordant phylogenetic relationships of 7312A *env* and *pol* sequences in the context of a single genetic unit.

Inspection of the deduced protein sequences indicated conserved as well as variable protein domains (Fig. 2). As expected, regions of known protein function were highly conserved. These included the p16/p27 cleavage site in the *gag* precursor (85), the WVPAHKGI/LGGN region in the RNase H domain (17), and the catalytic YMDD domain in the RT (51, 90). Also conserved was the spacing of histidine, cysteine, aspartic acid, and glutamic acid residues (HHCC and D, D-35-E motifs), known to be essential for integrase function (9, 89), as well as two cysteine residues in the exterior portion of gp41 which are believed to form an apical loop (30). Finally, there were no length differences except in the *gag* region (flanking the p16/p26 cleavage site), where a subset of viruses (ON, FT, JA, 7312A, 7810A, and 60667K) had the same codon deletions relative to the consensus sequence.

Phylogenetic analyses. To determine the evolutionary relationships of the newly identified HIV-2 strains to previously reported HIV-2 and SIV_{SM}/SIV_{MAC} isolates, phylogenetic trees were constructed from the PCR-derived *gag*, *pol* (integrase and RT), and *env* sequences. As shown in Fig. 3, these analyses revealed five distinct phylogenetic lineages, or sequence subtypes, of HIV-2. Two of these corresponded to the previously defined subtypes A and B, while three others represented new subtypes of HIV-2. Importantly, the grouping of viruses within the different lineages was consistent among trees from all four genomic regions; that is, viruses that belonged to a particular lineage in *gag* (Fig. 3A) also clustered within the same lineage in *pol* (Fig. 3B and C) and *env* (Fig. 3D). As noted previously (32), there was only one clear exception: 7312A clustered in different lineages depending on which parts of its genome were analyzed. In phylogenetic trees derived from *gag* and *pol* (RT and integrase) sequences, it clustered with subtype B viruses. However, in trees constructed from *env* sequences, it clearly grouped within subtype A. Since both PCR-derived (32) and proviral sequences (this study) exhibited the same discordant branching order, we concluded that 7312A contains a mosaic genome generated by recombination of subtype A and B viruses.

Three of the newly characterized viruses (FA, 60415K, and 7942A) fell within the group that is known as subtype A. This group, which contained the greatest number of viruses, was clearly defined regardless of whether *env*, *pol*, RT, or *gag* sequences were compared and occurred in 100% of bootstraps in all four trees. Analysis of the increased number of subtype A viruses also revealed an additional level of phylogenetic structure within this group, i.e., two distinct subclusters became apparent in three of the four trees. One subset of viruses, including BEN, D194, MVP-15132, and GH1, consistently grouped separately from other subtype A strains. The other viruses, represented by ROD, ISY, ST, CAM2, and NIH₂, also formed a monophyletic group in the *env*, *pol* (RT), and *gag* trees but not in the *pol* (integrase) tree. Interestingly, one virus (FA) for which three of the four regions were sequenced fell into both subclusters. In the *gag* and *pol* (integrase) regions, FA grouped with BEN, GH1, D194, and MVP-15132, but in the *env* region, it grouped with ROD, ST, NIH₂, ISY, and CAM2. These discordant relationships were supported by high bootstrap values and suggested a second example of a mosaic genome, this time involving recombination of divergent viruses from the same sequence subtype.

Five other newly characterized strains (ON, FT, JA, 7810A, and 60667K) clustered with D205 and UC1 in what has been termed subtype B. Again, this cluster emerged with high bootstrap values in trees from all four genomic regions. Two of the new viruses (ON and FT) were particularly closely related, differing by only 4% in their PCR-derived *gag* region. Since subjects ON and FT were husband and wife, we deduce that one infected the other by heterosexual transmission.

The three other viruses (2238 and FO784 from Liberia and PA from Sierra Leone) each defined a new lineage, and thus a

FIG. 2. Alignment of partial *gag* (p16/p28), *pol* (RT and RNase H/integrase region), and *env* (gp41 region) protein sequences from 12 independent HIV-2 strains. Nucleotide sequences were determined from PCR amplification products (cloned into plasmid or M13 vectors), translated, aligned, and compared with the HIV-2/SIV_{SM}/SIV_{MAC} consensus sequence from the database (65). Dashes denote sequence identity with the consensus sequence, and dots represent gaps introduced to optimize the alignments. Capital letters in the consensus sequence indicate sequence identity among all HIV-2 strains in the database. Lowercase letters and question marks indicate sites at which >50 or <50% of viruses have the same amino acid residue, respectively. Boxed amino acid residues and arrows above the consensus sequence indicate domains known to be important for protein function. The p16/p26 *gag* precursor cleavage site as well as the beginning of the membrane-spanning domain in gp41 are indicated. Asterisks denote previously published FO784 and 2238 sequences (32), and the symbol highlights 7312A sequences derived from a single, replication-competent provirus (37).

Phylogenetic tree showing the relationships between HIV-2 and SIV strains. The tree is rooted on the left and branches to the right. Bootstrap values are indicated at the nodes. Strains are grouped into five major clusters labeled B, C, A, D, and E. A scale bar at the bottom indicates 5% divergence.

Strains and their bootstrap values (from top to bottom):

- HIV-2_{7312A} (100)
- HIV-2_{60667K} (100)
- HIV-2_{D205}
- HIV-2_{UC1}
- HIV-2_{ON} (100)
- HIV-2_{FT} (100)
- HIV-2_{JA} (100)
- HIV-2_{7810A}
- HIV-2₂₂₃₈
- HIV-2_{BEN} (99)
- HIV-2_{GH1} (95)
- HIV-2_{FA} (95)
- HIV-2_{D194}
- HIV-2_{CAM2} (100)
- HIV-2_{7924A} (95)
- HIV-2_{ROD} (95)
- HIV-2_{60415K} (94)
- HIV-2_{NIHz}
- HIV-2_{ST}
- HIV-2_{ISY}
- SIV_{STM37.16} (100)
- SIV_{STM22579}
- HIV-2_{FO784} (100)
- SIV_{SMH4} (100)
- SIV_{SMPBj} (100)
- SIV_{MNE} (100)
- SIV_{MAC142}
- SIV_{MAC251}
- HIV-2_{PA}

Scale bar: 5% divergence

Phylogenetic tree showing the relationships between HIV-2 and SIV sequences. The tree is rooted at the bottom left. The sequences are grouped into five main clades labeled A, B, D, and E. Bootstrap values are indicated at the nodes. A scale bar indicates 5% divergence.

- Clade B:** HIV-2_{T312A} (100), HIV-2_{D205} (100), HIV-2_{UC1} (100).
- Clade A:** HIV-2_{BEN} (86), HIV-2_{GH1} (100), HIV-2_{D194} (100), HIV-2_{CAM2} (100), HIV-2_{ROD} (100), HIV-2_{S52907} (100), HIV-2_{NIHz} (100), HIV-2_{ST} (100), HIV-2_{ISY} (100).
- Clade D:** SIV_{STM37.16} (98), HIV-2_{FO784} (100), SIV_{SMH4} (100), SIV_{SMPBj} (98), SIV_{MNE} (100), SIV_{MAC142} (93), SIV_{MAC251} (93).
- Clade E:** HIV-2_{PA} (100).

5% divergence

Phylogenetic tree showing the relationships between HIV-2 and SIV sequences. The tree is rooted on the left and branches to the right. Bootstrap values are indicated at the nodes. Sequences are grouped into four main clusters labeled A, B, C, and D on the right. Cluster B includes HIV-2_7312A, HIV-2_D205, HIV-2_UC1, HIV-2_ON, and HIV-2_JA. Cluster C includes HIV-2_2238*. Cluster A includes HIV-2_BEN, HIV-2_GH1, HIV-2_FA, HIV-2_D194, HIV-2_MVP-15132, HIV-2_CAM2, HIV-2_ROD, HIV-2_NIH2, HIV-2_ST, and HIV-2_ISY. Cluster D includes HIV-2_F0784*, SIV_STM37.16, SIV_SMH4, SIV_SMPBJ, SIV_SMLIB, SIV_MNE, SIV_MAC142, and SIV_MAC251. A scale bar at the bottom indicates 5% divergence.

Phylogenetic tree showing the relationships between HIV-2 and SIV strains. The tree is rooted on the left and branches to the right. Bootstrap values are indicated at the nodes. Strains are grouped into four major clades labeled A, B, C, and D on the right. Clade B includes HIV-2D205, HIV-2GH2, HIV-2UC1, HIV-2ON, and HIV-2JA. Clade C includes HIV-22238 and HIV-2BEN. Clade A includes HIV-2GH1, HIV-2MVP-15132, HIV-2D194, HIV-2ROD, HIV-2CAM2, HIV-2NIH, HIV-2GB1222, HIV-2FA, HIV-27312A, HIV-2ST, and HIV-2ISY. Clade D includes SIVSTM37.16, SIVSTM22579, SIVSMH4, SIVSM P209, SIVSM B670, SIVSM PBj, HIV-2FO784, SIVMNE, SIVMAC142, and SIVMAC251. A scale bar at the bottom left indicates 5% divergence.

TABLE 4. Intra- and intercladal distances among sequence subtypes of HIV-2 and HIV-1^a

HIV-2 sub-type	% Nucleotide diversity					HIV-1 sub-type	% Nucleotide diversity				
	A	B	C	D	E		A	B	C	D	O
A	8.8					A	9.3				
B	17.3	10.0				B	12.8	4.0			
C	18.2	18.6	N/A			C	14.2	11.3	6.4		
D	17.6	16.7	18.4	N/A		D	13.6	8.0	10.8	6.5	
E	24.6	23.9	23.4	24.6	N/A	O	30.9	31.6	31.9	31.5	10.5

^a Average intra- and intercladal distances were calculated for the PCR-derived HIV-2 *gag* sequences and compared with values obtained for corresponding HIV-1 *gag* sequences from the database (65). Sequences were aligned by using MASE (26), and distances were calculated by using DOTS (55). Intracodal distances for HIV-2 subtypes C, D, and E are not available (N/A), since only single representatives have thus far been identified.

new sequence subtype, of HIV-2. Virus 2238 consistently appeared as a highly divergent strain, regardless of whether *gag*, *pol* (integrase), or *env* fragments were analyzed (2238 is not included in the *pol*-RT tree because this fragment could not be amplified). In trees derived from *pol* (integrase) and, particularly, *env* sequences, 2238 clustered relatively more closely with subtype B than with subtype A viruses, but in the *gag* tree, this situation was reversed. Since the latter grouping was found in only a small number of bootstrap samples (33%), this discrepancy was not significant. Moreover, branch lengths for 2238 were very deep in trees from all three different genomic regions. For these reasons, we defined 2238 as the first member of a third lineage, termed sequence subtype C.

As reported previously, FO784 clustered with simian viruses infecting sooty mangabeys and macaques rather than with viruses of human derivation (32). This was the case in trees from all four genomic regions, including the newly constructed *gag* tree. The precise branching order within the FO784/SIV cluster and the number of bootstrap replicates supporting it as an independent group varied somewhat among the four different trees. However, in the *pol* (RT) tree, which is derived from the longest sequence and thus likely the most reliable, the FO784/SIV cluster appeared in 98% of bootstraps. Interestingly, the phylogenetic depth within the FO784/SIV cluster was significantly greater than that within subtype A or B. Because of this and the extensive divergence from subtype A, B, and C viruses, we designated FO784 as a representative of a fourth major subtype (subtype D; note that we use HIV-2 subtype designations only for viruses of human derivation [82]).

The most divergent of all the HIV-2 strains was the virus infecting subject PA. In the two genomic regions for which sequence was available, PA formed the first lineage to branch off from this group. Moreover, branches separating this point from the main cluster were quite long, indicating an earlier divergence of PA than of the other members of the HIV-2/SIV_{SM}/SIV_{MAC} family. Because of this and its overall distance from subtype A, B, C, and D viruses, we designated PA as a representative of sequence subtype E.

Apart from the observation that subtype E (PA) branched off first, there was no clear order of branching among the other subtypes. Thus, subtypes A, B, and C clustered, to the exclusion of the subtype D/SIV lineage, in two of the four trees (*gag* and *env*) but not in the other two. Furthermore, the branches outside these clusters were very short. These results are consistent with a star phylogeny and suggest that subtypes A to D diverged at roughly the same time. Very similar results were obtained with the dynamically (asymmetrically) weighted parsimony method, which produced trees that were virtually identical to those shown in Fig. 3 in both topology and branch lengths (data not shown).

Serological profile of sera from individuals infected with the five different HIV-2 subtypes. From the phylogenetic analyses, it was clear that the virus infecting subject PA represented the most divergent HIV-2 strain identified thus far. In the PCR-derived *gag* region, PA differed from all other HIV-2 strains in 25% of its nucleotide sequence, while members of HIV-2 subtypes A to D differed from each other by less than 19% (Table 4). In the same genomic region, members of the highly divergent group O differed from all other HIV-1 strains in 30% of their nucleotide sequence, while viruses of HIV-1 subtypes A to D differed by less than 14% (Table 4). Since subgroup O infections, because of their genetic distance, are not detected by all commercially available serological assays (59), we wanted to know whether this was also the case for the divergent subtypes of HIV-2. Serum (or plasma) samples from all study subjects were thus analyzed side by side with three commercially available and widely used confirmatory tests (the Pasteur NEW LAV-BLOT II and PEPTI-LAV 1-2 assays, as well as the Cambridge Biotech HIV-2 Western blot kits). The results revealed comparable serological profiles for all samples (Figure 4 illustrates results obtained with the Cambridge Biotech HIV-2 Western blot kit). Most sera, including those of FO784 (subtype D) and PA (subtype E), reacted strongly with all HIV-2 antigens. Weak reaction with the major capsid protein p26 was observed with three sera (including 2238), but this did not affect their scoring as HIV-2 antibody positive. In addition, all sera reacted strongly in the PEPTI-LAV 1-2 assay, indicating sufficient cross-reactivity even with this peptide-based antigen (data not shown). Finally, cross-reactivity with HIV-1 antigens was also observed (Table 1). However, this dual seroreactivity was not correlated with any particular subtype. Taken together, these results indicate that currently available commercial serological assays recognize infection with all five HIV-2 subtypes with comparable efficiency.

DISCUSSION

Identification of five distinct sequence subtypes of HIV-2.

The purpose of this study was to investigate the extent of HIV-2 genetic diversity among 12 newly identified HIV-2 strains by direct analysis of viral sequences in uncultured patient tissue and to examine their evolutionary relationships by comparison to previously characterized HIV-2 and SIV_{SM}/SIV_{MAC} sequences. PCR amplification of four independent

FIG. 3. Phylogenetic trees of HIV-2 subtypes derived from DNA sequences of partial *gag* (A), *pol* RT (B), *pol* integrase (int) (C), and *env* (D) genes. Phylogenetic relationships were determined by the neighbor-joining method as described in Materials and Methods. Horizontal branch lengths are drawn to scale, while vertical branches are for clarity only. The numbers on the nodes represent the percentage of bootstrap samples with which the cluster to the right is supported; only those values greater than 80% are shown. The trees were rooted by using SIV_{MND} as an outgroup (65). Brackets denote individual sequence subtypes as described in Results. Newly characterized HIV-2 strains are boxed; viruses shown in white on black failed to grow in tissue culture. Asterisks indicate previously published sequences from FO784 and 2238 (FO784*pol*.C12, M87110; 2238*pol*.B7, M87138; FO784*env*.A13, M87069; 2238*env*.B10, M87118; and FO784*RT*.C2, M87111 [32]). Dynamically weighted parsimony methods (95) yielded virtually identical trees (with respect to both topology and branch lengths).

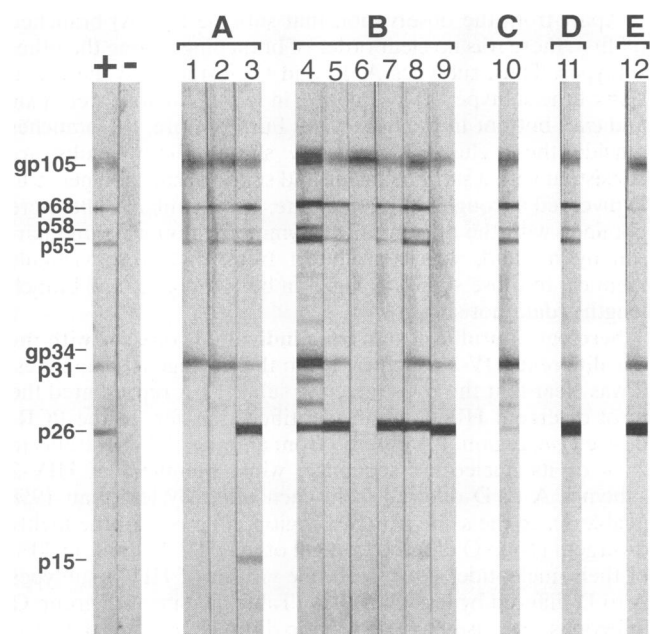


FIG. 4. Western blot profiles of sera from individuals infected with the five sequence subtypes of HIV-2. Serum (or plasma) samples from subjects FA (lane 1), 7924A (lane 2), 60415K (lane 3), 7312A (lane 4), 60667K (lane 5), ON (lane 6), FT (lane 7), JA (lane 8), 7810A (lane 9), 2238 (lane 10), FO784 (lane 11), and PA (lane 12) were analyzed for HIV-2 seroreactivity with the Cambridge Biotech HIV-2 Western Blot kit. Lanes + and -, positive and negative serum controls, respectively, supplied by the manufacturer. Immunoreactive HIV-2 proteins are indicated. Sera from individuals infected with viruses from the same sequence subtype are grouped.

and nonoverlapping genomic regions allowed a comprehensive analysis of HIV-2 phylogeny and identified five distinct and roughly equidistant genetic lineages which, by analogy to HIV-1, have been termed sequence subtypes A to E (Fig. 3). Importantly, the majority of the newly identified viruses failed to replicate in tissue culture, and their molecular characteristics would have gone unrecognized if samples had been subjected to *in vitro* cultivation prior to sequence analysis (as is customary for HIV-1 and HIV-2). Moreover, one of the culture-negative viruses (PA) was found to differ in 25% of its nucleotide sequence in the *gag*, *pol*, and *env* regions from previously reported HIV-2 strains, which approaches the levels of divergence observed between the two major groups of HIV-1 (groups M and O). These data thus indicate that the extent of HIV-2 genetic diversity is comparable to that of HIV-1 and that multiple sequence subtypes exist for both viruses. In contrast to HIV-1, however, tissue culture of HIV-2 appears to be highly selective and to amplify only a subset of naturally occurring viruses. PCR amplification of viral sequences from primary patient material thus seems to represent a more reliable method of screening for genetic variants of HIV-2.

Geographic distribution of the various HIV-2 sequence subtypes. The identification of five independent HIV-2 lineages also allowed an analysis of their prevalence and geographic distribution. Previous studies found no apparent correlation between the geographic origin of an HIV-2 isolate and its phylogenetic clustering (8, 65). In particular, subtype A viruses were identified in many diverse locations all across West Africa, including Senegal (ST and MVP-15132), the

Cape Verde Islands (ROD), Mali (BEN), Ghana (GH1), the Gambia (ISY and D194), and Guinea Bissau (NIH₂, CAM2, and GB1222). Analyses of the 12 new viruses confirmed these earlier observations, demonstrating in addition a lack of geographic clustering among HIV-2 strains representing sequence subtypes B to E. The three newly identified subtype A viruses came from individuals who originated from Ghana, Guinea Bissau, and Senegal. The five new subtype B viruses were derived from subjects most likely infected in Ghana, Côte d'Ivoire, Sierra Leone, and possibly Nigeria (subject JA). Finally, the single representatives of subtypes C (2238) and D (FO784) were identified in Liberia (32), while the representative of subtype E (PA) likely came from Sierra Leone (43). Thus, the origins of HIV-2 strains of different subtypes, particularly those of members of the well-represented sequence subtypes A and B, overlapped considerably. This is consistent with the finding of intersubtype recombinants (see below), which require the presence and simultaneous spread of divergent viral strains in the same population.

Simultaneous coinfection and viral recombination in HIV-2-infected individuals. As shown in Fig. 3, the grouping of the various HIV-2 strains into different sequence subtypes was consistent across all four genomic regions analyzed. Nevertheless, there were two viruses which fell in different phylogenetic positions depending on which parts of their genomes were compared (in both cases, the discordant branching orders were supported by greater than 99% of bootstrap values and were thus highly significant). Virus 7312A clustered with subtype B viruses in the *gag* and *pol* (RT and integrase) regions but grouped with subtype A viruses in the *env* tree. Since all 7312A sequences were derived from the same proviral clone, these results indicate that it contains a mosaic genome and represents a recombinant of viruses belonging to sequence subtypes A and B. Similarly, FA fell into one subtype A subcluster in *gag* and *pol* but grouped with a different subcluster in the *env* region. This discordant branching may also be due to a mosaic (recombinant) genome or, alternatively, may indicate simultaneous coinfection of patient FA with two divergent subtype A viruses which were differentially amplified by the *pol* and *env* primer pairs. Cloning of a complete FA provirus will be necessary to distinguish between these two possibilities. Regardless of which is the case, these examples document that individuals can be coinfecting with divergent HIV-2 strains.

Although it is presently unknown how and under what circumstances the same person becomes superinfected with two different viruses, it is of interest that similar findings have also been reported for HIV-1. One of the earliest characterized African strains (MAL [2]) contains a mosaic genome comprising sequences from subtype D and A viruses (56, 82), and more recently characterized isolates from Thailand might represent recombinants of viruses belonging to sequence subtypes E and A (60, 82). These results thus indicate that superinfection and recombination of phylogenetically distinct viruses can occur in both HIV-1- and HIV-2-infected individuals. Moreover, on the basis of our results with HIV-2, these events may happen relatively frequently (2 of 22 HIV-2 strains in Fig. 3 exhibit a discordant branching order). Systematic studies are needed to determine exactly how often and under what conditions individuals acquire genetically divergent HIV-1 or HIV-2 strains, since this information may be important to current AIDS vaccine development efforts (particularly in Africa, where numerous HIV-1 and HIV-2 sequence subtypes circulate in the same populations).

Phylogenetic evidence for multiple introductions of genetically diverse sooty mangabey viruses into human and macaque populations. In addition to defining new HIV-2 subtypes, the

phylogenetic trees in Fig. 3 also shed new light on our understanding of the origins and molecular evolution of the entire HIV-2/SIV_{SM}/SIV_{MAC} group of viruses. For a number of reasons, it is now believed that the common ancestor of this group infected sooty mangabeys and that HIV-2 in humans and SIV_{MAC} in macaques are each the result of cross-species transmissions. Most importantly, significant numbers (approximately 30%) of sooty mangabeys are infected with SIV_{SM} in the wild (63). By contrast, SIV_{MAC} has not been found in wild-living Asian macaques but has been identified in four different macaque species living in captivity (16, 48, 69). Also, SIV_{SM} is nonpathogenic in sooty mangabeys (29, 42, 64) but is highly pathogenic in macaques (16, 48, 69), as is the case for many zoonotic infections that cause less severe or no disease in their natural host (87). From these findings, it thus seems clear that SIV_{MAC} was generated inadvertently by transfer of SIV_{SM} from naturally infected sooty mangabeys to macaques in the setting of primate centers. Similarly, naturally infected sooty mangabeys also represent the probable source of HIV-2 in the human population. The natural habitat of sooty mangabeys coincides with the geographic pattern of HIV-2 endemicity in West Africa (32, 64), and in many West African countries, mangabeys are hunted for food and kept as pets (63, 64). Thus, scratches and bites of humans by monkeys and exposure to monkey blood in the context of food preparation are commonplace. In addition, accidental transmission of an SIV_{SM} strain to a laboratory worker has recently demonstrated that humans are susceptible to infection with sooty mangabey viruses (47). Taken together, these observations strongly indicate that naturally infected sooty mangabeys represent the reservoir for this entire group of viruses.

The phylogenetic relationships of the various HIV-2, SIV_{SM}, and SIV_{MAC} lineages shown in Fig. 3 thus suggest that sooty mangabey viruses have entered both human and macaque populations on more than one occasion. For example, the two distinct and highly divergent clusters of SIV_{STM} (from stump-tailed macaques) and SIV_{MAC}/SIV_{MNE} (from rhesus and pig-tailed macaques) probably arose through independent SIV_{SM} transmission events. Assuming that the ancestral virus (at the root at the left side of Fig. 3) infected sooty mangabeys, then each of the HIV-2 lineages leading to PA, FO784, and the A subtype must have similarly arisen from independent mangabey-to-human transfers. Moreover, the deep divergences of 2238 and subtype B viruses may also reflect independent SIV_{SM} transmissions. Then, a minimum of three and perhaps as many as five instances (i.e., one for each sequence subtype) of mangabey-to-human transmission are necessary to explain the phylogeny of currently known HIV-2 sequences.

The conclusion that HIV-2 evolved as a result of cross-species transmission from naturally infected sooty mangabeys raises the questions of why all SIV_{SM} isolates cluster within only one of the five major lineages in Fig. 3 and why the earliest-diverging branch is represented by a human virus (PA). Although currently available sequence information is still limited, there are several lines of evidence suggesting that the genetic diversity of naturally occurring SIV_{SM} strains is far greater than presently appreciated. First, all but one of the SIV sequences in Fig. 3 are derived from captive monkeys (macaques and sooty mangabeys) housed in American primate centers (the only "natural" SIV in the subtype D/SIV cluster is SIV_{SM}L1B, which was isolated from a Liberian pet sooty mangabey [64]). It is thus likely that the SIV lineages defined in Fig. 3 represent only a limited number of SIV_{SM} introductions, possibly involving sooty mangabeys captured in the same geographic area (interestingly, the human FO784 strain, which clusters in the same group, also originated from Liberia [32]).

Second, all currently available SIV_{SM}/SIV_{MAC} sequences are derived from viral isolates propagated in immortalized human T-cell lines prior to molecular analysis. Given the obvious selection biases of tissue culture with respect to HIV-2 isolation (see below), it is quite possible that these SIV isolates also represent only a subset of viruses inadvertently selected because of their in vitro growth characteristics. Finally, two groups of investigators have recently reported phylogenetic data for additional sooty mangabey strains which indicate that they branch outside the subtype D/SIV cluster (11, 71). Taken together, these findings suggest that there may be many more SIV_{SM} lineages than currently identified and that analysis of wild-living sooty mangabeys and PCR amplification of uncultured animal material will be necessary to determine the full extent of their genetic diversity.

Subtype-specific differences in virus biology. Several previous studies noted lower isolation frequencies for HIV-2 than for HIV-1 (1, 81, 83). Culturing virus from 20 HIV-2 seropositive individuals from the Gambia, Schulz and coworkers recovered only seven isolates (81). Similarly, Albert et al. described lower isolation frequencies for HIV-2, particularly from individuals with asymptomatic infection (1). Finally, Simon and coworkers compared HIV-1 and HIV-2 isolation frequencies systematically and reported a strong correlation between HIV-2 recovery and clinical status (83). HIV-2 was isolated from 100% of patients with CD4 counts below 200/ μ l (10 of 10), from 62% of patients with CD4 counts between 200/ μ l and 500/ μ l (5 of 8), and from 18% of patients with CD4 counts above 500/ μ l (4 of 22). By contrast, HIV-1 was isolated from virtually all patients (52 of 54) at all stages of infection (100, 94, and 92% recovery rates, respectively). In agreement with these earlier studies, we also noted a markedly reduced ability to culture HIV-2 from our patients. Only five HIV-2 isolates were obtained from the 12 seropositive subjects despite repeated attempts in several independent laboratories. However, the strongest predictor of in vitro viral growth in our study was whether a virus was a member of a particular sequence subtype. All subtype A viruses yielded positive cultures regardless of whether they were derived from asymptomatic individuals with high CD4 counts (60415K) or from blood samples transported for extended periods of time (FA). Even the recombinant 7312A virus, which contains a subtype A *env* gene in the context of a subtype B genome (37), was recovered on three independent occasions from an individual with CD4 counts above 500 cells per mm³ (Tables 1 and 2). By contrast, isolation frequencies from individuals infected with subtype B to E viruses were significantly lower. Only one of five subtype B viruses (60667K, from a patient with reduced CD4 counts) yielded a positive culture, despite the fact that two additional individuals (ON and JA) with subtype B infections suffered from end-stage AIDS. Finally, none of the single representatives of sequence subtypes C, D, or E replicated in vitro. This was the case despite several different isolation strategies, particularly for subject PA, which included cocultivation with different target cells and depletion of patient CD8 cells, which have been reported to produce a factor(s) that suppresses viral replication (91). These results thus suggest differential abilities of members of the various HIV-2 subtypes to grow under standard tissue culture conditions.

Simon and coworkers also reported a correlation between HIV-2 isolation frequencies and virus load in infected individuals (83). HIV-2-infected subjects with CD4 counts greater than 200/ μ l harbored considerably less virus (as determined by limiting-dilution cultures as well as semiquantitative DNA PCR) than did patients infected with HIV-1. It was thus proposed that the ability of an HIV-2 strain to grow in vitro

was also an indicator of its *in vivo* replication potential. Given our finding of higher isolation frequencies for subtype A viruses, the question arises of whether subtype A viruses grow to higher titers *in vivo* as well as *in vitro* and thus exhibit a more virulent phenotype. In this context, it is important to point out that subtype A viruses are numerous and prevalent throughout West Africa (65). Moreover, subtype A viruses are the only HIV-2 strains that have been identified in large numbers outside of West Africa (33). Several recent reports indicate that they are spreading epidemically in India, where they may be associated with accelerated disease (73, 77). Also, sequences of subtype A viruses are the most common in the database (65), which is an additional indicator of their wide distribution, efficient transmission, and propensity to grow in culture. In light of these data, it is tempting to speculate that there are subtype-specific differences in HIV-2 biology and that certain naturally occurring strains replicate to higher titers, transmit more efficiently, and cause more virulent infections. Systematic natural history studies are needed to determine whether this is the case and whether there are subtype-specific differences in HIV-2 pathogenicity. Also, the mechanisms responsible for the observed *in vitro* differences, which may include variation in cell tropism, potency of regulatory proteins, quasi-species complexity, proportion of defective viruses, or some combination of these factors, will have to be elucidated. This information is crucial not only to ongoing HIV drug and vaccine development efforts, but also for a better understanding of the virologic mechanisms underlying AIDS pathogenesis.

As important as evaluation of virulent HIV-2 strains will be studies characterizing infections with viruses such as FO784, 2238, and PA. Their resistance to growth in tissue culture, limited geographic distribution, and derivation from healthy individuals and communities where HIV-2-associated AIDS has not yet been recognized (32, 43) suggest relatively inefficient transmission and low pathogenic potential. As HIV-1-infected persons are monitored over time, it has become apparent that there are marked differences in the course of their disease progression. Whereas the mean period to development of symptomatic disease is approximately 10 years, cohort studies have identified persons who have been infected for up to 15 years with no evidence of CD4 decline (58). These individuals are seropositive and contain HIV-1 sequences in their blood cells but are generally virus isolation negative, presumably because of a low viral burden. Subjects FO784, 2238, and PA described in this study mimic many of these properties and could thus represent long-term survivors of HIV-2. In particular, subject PA fits this description, since this Sierra Leonean immigrant was infected with HIV-2 for probably 20 years (43) and never exhibited CD4 decline or any other manifestation of HIV-2 disease (he eventually died of causes unrelated to his HIV-2 infection). Also, it is clear that lentiviral virulence properties are dictated by a complex interplay between both viral and host determinants and that introduction of a virus into a new host species and serial passage through members of the same species represent contributing factors to disease progression. Given that some of the various HIV-2 subtypes (e.g., FO784 and PA) have likely resulted from independent transmissions of sooty mangabey viruses, the question arises whether they differ in their level of adaptation, and thus growth potential, in the human host. Cohorts of individuals like subjects 2238, PA, and FO784 should be identified to establish the long-term effects of their HIV-2 infections and to determine whether they harbor truly attenuated viruses with reduced *in vivo* replication potential.

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